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Monoclonal antibody CI-panHu defines a pan-human cell-surface antigen unique to higher primates

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SUMMARY

The murine monoclonal antibody CI-panHu reacts strongly with the cell surface of all human cells, including erythrocytes, tumour cells and HLA-A,B,C-negative cell lines. As such, this antibody defines the first pan-human cell-surface antigen reported. The antigenic determinant detected is associated with a protein doublet of 16,000 MW whose expression is restricted to cells from humans, apes and some species of Old World monkeys. Antibody reactivity is not diminished by routine fixation procedures, nor by paraffin-embedding, and the antigenic determinant is relatively protease-resistant. The use of this antibody as a positive control in immunoassays of human cells is discussed.

INTRODUCTION

A major use of many monoclonal antibodies is in immunoassays performed on intact human cells or on human tissue sections. A key requirement in any immunoassay is a positive control. There is, therefore, a need for a suitable, and preferably monoclonal, antibody to serve as positive control for each tissue tested. In particular, there is an absolute requirement for positive control antibodies when the tissue distribution and cell specificity of monoclonal antibodies are assessed by immunohistochemical techniques. In such experiments it is not justifiable to score negative reactions except in the presence of at least one positive reaction. This is a necessary precaution to confirm adequate tissue preservation and functioning technology. In most instances where human cells comprise the target tissue, anti-HLA-A,B,C monoclonal antibodies are used as positive controls. However, there are a number of human cell types in which HLA-A,B,C antigen expression is either low or absent (Gotze, 1977; Brodsky, Bodmer & Parham, 1979; Rowe & Beverley, 1984). Also, HLA-A,B,C antigenic determinants, in common with many other determinants detected by monoclonal antibodies, exhibit poor and variable survival when subjected to the fixation procedures employed in routine pathology laboratories.

In this paper, we report the production and characterization of a monoclonal antibody CI-panHu which reacts strongly with the surface of all human cells. This antibody reacts with a protein doublet of 16,000 MW, as well as a minor component of 13,000 MW. The CI-panHu antigenic determinant survives all fixation procedures tested and is relatively protease-resistant. The expression of this determinant is more restricted phylogenetically than monomorphic HLA Class I determinants (Parham, Sehgal & Brodsky, 1979), being detected in cells of humans, apes and some species of Old World monkey. The use of this antibody in immunofluoresence and immunoperoxidase staining procedures is demonstrated. This antibody has been successfully used as a positive control reagent for assessment of the distribution of monoclonal antibody-defined antigenic determinants in the human (de Kretser *et al.*, 1985).

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MATERIALS AND METHODS

Cells

Normal blood and bone marrow samples were obtained from healthy donors. Mononuclear cells were separated by Ficoll density gradient centrifugation (Boyum, 1964) and immediately tested. Thymocyte and lymph node cell suspensions were prepared by teasing fresh tissue into buffered saline. Established cell lines used in this study were grown in suspension or monolayer culture in RPMI-1640 medium (Flow Laboratories, North Ryde, Australia), supplemented with 10% fetal calf serum (Flow Labs) and 40 μg/ml gentamycin (Roussel Pharmaceuticals, Castle Hill, Australia).

Hybridoma production

BALB/c mice were initially immunized intraperitoneally with 10^7 viable Molt 4 (T-cell line) cells and boosted intravenously 3 weeks later with 10^7 viable cells. After 4 days, the spleen from an immunized mouse was removed, the splenocytes fused with the

P3-NSl/l-Ag 4.1 (NSl) mouse myeloma, and hybrids propogated (Brodsky, Parham & Bodmer, 1980). The hybrid CI-panHu was selected on the basis of its reactivity with cell lines (see below) and was cloned by repeated successive limiting dilution (Lefkovits & Waldman, 1979).

Purification of CI-panHu antibody

The cloned hybrid CI-panHu was injected intraperitoneally into BALB/c mice (10^6cells/mouse) and ascitic fluid collected 14–28 days later. The monoclonal antibody was purified from the ascitic fluid by ammonium sulphate precipitation (Heide & Schwick, 1978) followed by protein-A affinity chromatography (Ey, Prouse & Jenkins, 1978). The elution pattern of the antibody from the protein-A column suggested that it was of the IgG1 subclass. This was confirmed by immunodiffusion (Ouchterlony, 1958) using antisera specific for the various mouse subclasses (Miles Labs, Mulgrave, Australia). The protein concentration of the active fraction was determined (Lowry *et al.*, 1951) and the purified antibody stored at −70° in aliquots at a concentration of 1 mg/ml. The maximum dilution of this 1 mg/ml stock solution of purified antibody which still gave maximal reaction by indirect immunofluoresence with the Molt 4 cell line was 10^{-3}. All results reported here were obtained using this purified antibody stock solution at a dilution of 10^{-2}.

Indirect immunofluorescence

The reactivity of the CI-panHu antibody with haematopoietic cell populations and all cell lines was determined by indirect immunofluorescence (Reinherz *et al.*, 1979). Cell samples (10^6 cells/test) were incubated for 5 min at 20° with 50 μl of test antibody, washed once in buffered saline and further incubated for 5 min at 20° with 50 μl of fluorescein-conjugated goat anti-mouse IgG $F(ab')_2$ fragment (Cappel Laboratories, West Chester, PA), diluted 1:20 in buffered saline. Cells were washed twice in buffered saline and fluorescence analysed on an Ortho System 50 cytofluorograph (Ortho Diagnostic Systems, North Ryde, Australia). The anti-HLA-A,B,C monoclonal antibody W6/32 (Seralab, Crawley Down, Sussex, U.K.), diluted 1:100, was tested for comparison in all assays. Normal mouse serum, diluted 1:100 in appropriate buffer, was used as negative control for all assays. Peripheral blood cell populations were separated using the 90° forward scatter and axial light loss parameters. The identity of separated populations was confirmed by indirect immunofluorescence using commercially available cell-type specific antibodies.

Immunohistochemistry

The reactivity of the CI-panHu antibody with cells in sections from various normal and malignant adult tissues and fetal tissues was assessed by a four-step immunoperoxidase staining technique (Hancock, Kraft & Atkins, 1982). Usually, solid tissues were either poly-L-lysine-fixed for frozen sections (Hancock *et al.*, 1982) or Bouin's-fixed paraffin-embedded routine pathology specimens (Drury & Wallington, 1967). Other fixation procedures tested included 10% formalin and poly-L-lysine fixation of frozen sections from snap-frozen tissue. Cytocentrifuge preparations (Shandon, London, U.K.) of normal blood and bone marrow samples were stained as above after formol-saline fixation (Barker, Silverton & Luckcock, 1966). Staining was also evaluated after ethanol, methanol, acetone or 10% formalin fixation.

Antigen stripping experiments

Aliquots of 10^6 cells were incubated at 37° for 1·5 hr in RPMI-1640 medium (no fetal calf serum) or in RPMI-1640 medium containing 1 mg/ml papain, pepsin, elastase, proteinase K, neuraminidase, subtilisin (all from Boehringer Mannheim, Melbourne, Australia), or trypsin (Worthington, Flow Labs). At termination of incubation, cell viability was greater than 90%. The binding of the CI-panHu antibody and the anti-HLA-A,B,C antibody W6/32 was assessed by indirect immunofluorescence immediately after cessation of incubation.

Biosynthetic labelling of cells and immunoprecipitation

Cells (10^7) of the Raji Burkitt lymphoma cell line were biosynthetically labelled with ^{35}S-methionine by 24 hr incubation at 37° in 75 cm^2 flasks in 10 ml methionine-free RPMI-1640 (Gibco, Melbourne, Australia) containing 10% dialysed fetal calf serum and 1 mCi ^{35}S-methionine (Amersham, Sydney, Australia). Cells were harvested by centrifugation, washed once, and resuspended in 10 ml buffered saline, pH 7·2. One-ml aliquots of labelled cells were dispensed into six tubes and the following antibodies added: 1 μl normal mouse serum (NIg; two tubes), 0·5 μl CI-panHu (1 mg/ml; two tubes) and 1 μl W6/32 (Seralab; two tubes). After incubation at 20° for 30 min, the cells in these six tubes were lysed by the addition of 1 ml lysis buffer A (0·5% NP-40, 0·5% sodium deoxycholate in 50 mM NaCl, 25 mM Tris-HCl pH 8·1) to three tubes (1 × NIg, 1 × CI-panHu and 1 × W6/32) and by the addition of 1 ml lysis buffer B (1% NP-40 in 50 mM NaCl, 25 mM Tris-HCl, pH 8·1) to the remaining three tubes. The lysates were cleared of cell debris by centrifugation, and each supernatant was treated with 1 μl rabbit anti-mouse immunoglobulin antiserum. After incubation at 20° for 30 min, antigen–antibody complexes were precipitated by adding 15 μl *Staphylococcus aureus* suspension (SaCI; heat-killed, formalin-fixed Cowan I strain; 20% by vol.) and subsequently treated as described previously (de Kretser *et al.*, 1982). The remaining 4 ml of labelled cell suspension were lysed by addition of 2 ml lysis buffer A, cell debris removed by centrifugation, and 0·5-ml aliquots of the resulting lysate were separately treated with the three antibodies as detailed above. After incubation at 20° for 30 min, the aliquots were treated with rabbit anti-mouse immunoglobulin and SaCI as described above. The antigen–antibody–SaCI pellets were stored at −70° prior to analysis.

Sodium dodecyl sulphate–polyacrylamide gel electrophoretic analysis (SDS–PAGE)

One dimensional SDS–PAGE analyses of immunoprecipitated proteins using 15% acrylamide slab gels were performed as described by de Kretser *et al.* (1982).

RESULTS

Reactivity with human haematopoietic cells and cell lines

The antibody CI-panHu reacted strongly with all cell populations in 54 samples of normal blood and bone marrow (Table 1). Immunoperoxidase staining of cytocentrifuge preparations of peripheral blood and bone marrow mononuclear cell fractions confirmed the strong and uniform reaction of the CI-panHu antibody with the cell surface of all cells present (Fig. 1a). By contrast, anti-HLA-A,B,C antibodies did not react with ery-

Table 1. Reactivity of antibody CI panHu with normal haematopoietic cells and cell lines

Cell type	No. samples	Reactivity* with: CI-panHu	Anti-HLA†
Normal haematopoietic cells			
Peripheral blood			
erythrocytes	12	+++	−
granulocytes	12	+++	+++
monocytes	12	+++	+++
lymphocytes	12	+++	+++
Thymocytes	3	+++	+
Lymph node cells	9	+++	++
Bone marrow mononuclear cells	30	+++	+
Cell lines			
EBV-transformed			
B-lymphoblastoid	8	+++	+++
Burkitt's lymphoma	2	+++	+++
Burkitt's lymphoma (Daudi)	1	+++	−
T-lymphoid	6	+++	+++
Non-T, non-B lymphoid	4	+++	+++
Monocytic	3	+++	+++
Myeloid	1	+++	+++
Erythroleukaemic	1	+++	−

* Reactivity assessed by indirect immunofluorescence analysed by cytofluorograph: (−) < 10%; (+) 10–40%; (++) 40–80%; (+++) > 80% cells staining.

† Monoclonal anti-HLA-A,B,C antibody W6/32.

throcytes and only reacted weakly with certain bone marrow cell populations (Table 1). On immunoperoxidase staining, the anti-HLA-A,B,C antibody reacted well with small mononuclear cells (e.g. lymphocytes), but gave very weak staining of larger cells (e.g. bone marrow blasts, polymorphonuclear leucocytes) (Fig. 1b).

The CI-panHu antibody reacted with all 31 EBV-transformed or leukaemia-derived cell lines tested, including the erythroleukaemia-derived cell line K562 and the Burkitt lymphoma-derived cell line Daudi, both of which are negative for cell-surface reaction with anti-HLA-A,B,C antibodies (Table 1).

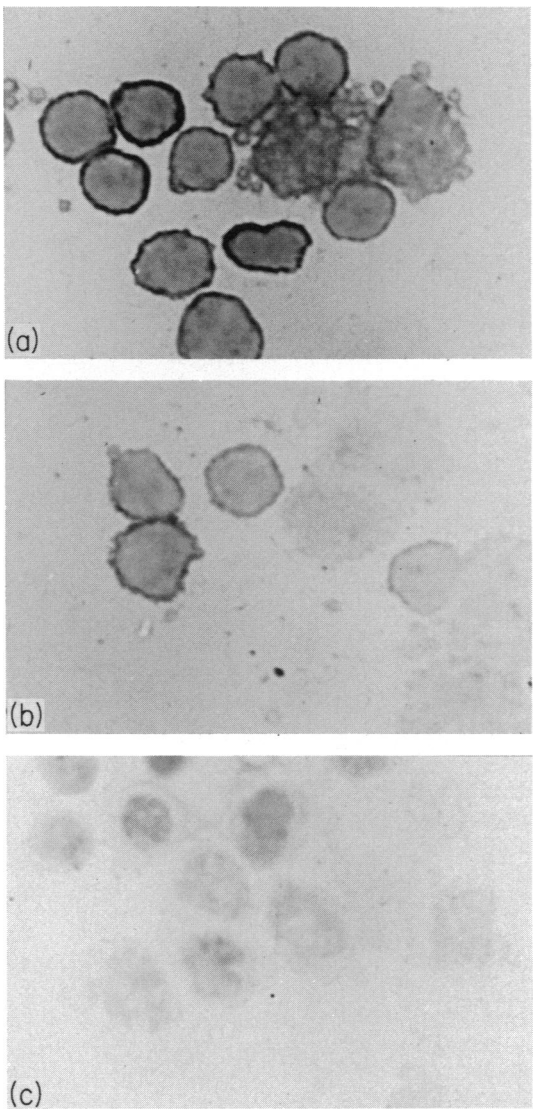

Figure 1. Immunoperoxidase staining of normal bone marrow mononuclear cells with antibody CI-panHu. Mononuclear cells were separated from peripheral blood from a healthy donor, cytocentrifuge preparations made and stained with antibodies CI-panHu (a), anti-HLA-A,B,C W6/32(b) and normal mouse immunoglobulin (c) by the immunoperoxidase procedure as described in the Materials and Methods. Methyl green was used as counterstain (magnification × 644).

Reactivity with normal human adult and fetal tissues

The CI-panHu antibody stained the cell surface of all cell types represented in 84 samples of 25 normal adult tissues (Table 2). In some instances, antigen was also detected intracellularly. The CI-panHu antibody reacted in a similar manner with all cell types in the seven fetal tissues tested (Table 2). Although HLA-A,B,C antigens could be detected in all tissues tested and in the majority of cell types, the intensity of staining achieved using the anti-HLA-A,B,C antibody W6/32 varied with cell type and was generally less than that obtained using the CI-panHu antibody.

Reactivity with human tumour tissues

The CI-panHu antibody, without exception, reacted with over 20 different types of tumour cell (Table 2). In contrast, the anti-HLA-A,B,C antibody W6/32 gave weak and patchy, or often negligible, reaction with many solid tumour cells. For example, on immunoperoxidase staining of tissue sections from a cervical endometrial carcinoma, the CI-panHu antibody showed strong reaction with the cell surface and moderate reaction with the cytoplasm of the tumour cells, as well as with the cell surface of other cell types present (Fig. 2a). In this tissue, reaction with the anti-HLA-A,B,C antibody was barely detectable with little or no staining of the tumour cells (Fig. 2b).

Fixation tolerance of CI-panHu antibody reactivity

The range of fixatives employed in the experiments reported

Table 2. Reactivity of antibody CI-panHu with normal adult fetal and solid tumour tissues

Tissue	No. samples	Reactivity* with CI-panHu
Normal adult		
Skin	10	+
Vagina	5	+
Cervix	6	+
Uterus	2	+
Ovary	4	+
Testis	2	+
Prostate	2	+
Bladder	2	+
Oesophagus	3	+
Stomach	3	+
Small intestine	4	+
Colon	4	+
Gall bladder	3	+
Pancreas	2	+
Thymus	2	+
Spleen	4	+
Lymph node	4	+
Tonsil	2	+
Liver	2	+
Lung	2	+
Heart	2	+
Kidney	5	+
Adrenal	1	+
Smooth muscle	4	+
Placenta	4	+
Fetal		
Skin	1	+
Thymus	1	+
Kidney	1	+
Small intestine	1	+
Large intestine	1	+
Blood vessels	1	+
Connective tissues	1	+
Solid tumours		
Breast adenocarcinoma	4	+
Melanoma	6	+
Colon carcinoma	2	+
Lymphoma	6	+
Astrocytoma	1	+
Stomach tumour	1	+
Parotid tumour	1	+
Vaginal SCC	2	+
Cervical SCC	6	+
Bladder TCC	1	+
Oesophagus SCC	1	+
Lung SCC	1	+
Skin SCC	5	+
Skin BCC	2	+
Ovarian adenocarcinomas	21	+
Ovarian fibrosarcoma	1	+
Uterine leiomyosarcoma	1	+
Cervical carcinomas	2	+
Metastatic carcinomas	10	+

* Reactivity assessed by indirect immunoperoxidase staining.

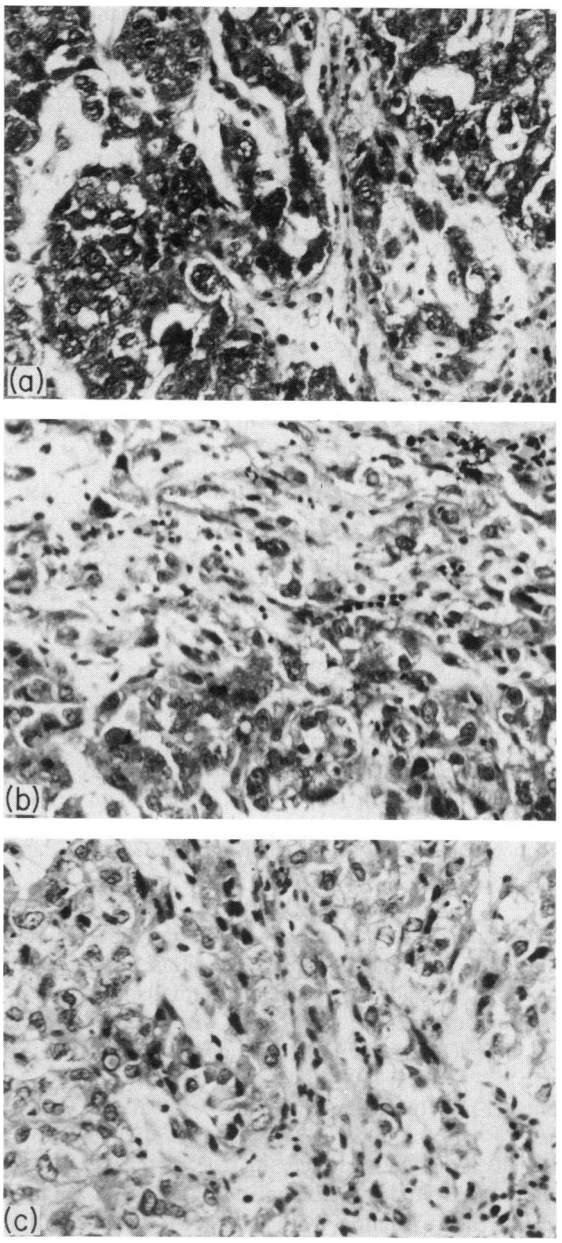

Figure 2. Immunoperoxidase staining of human tumour tissue with antibody CI-panHu. Serial sections from a Bouin's-fixed, paraffin-embedded sample of cervical endometrial carcinoma were stained with antibodies CI-panHu(a), anti-HLA-A,B,C W6/32(b) and normal mouse immunoglobulin (c) by the immunoperoxidase technique described. Gill's haematoxylin was used as counterstain (magnification × 150).

above encompasses the majority of fixation procedures routinely used in both research and clinical pathology laboratories. The antigenic determinant recognized by the CI-panHu antibody did not exhibit any fixation sensitivity.

Species specificity of antibody CI-panHu

Of the species tested, strong reactivity with antibody CI-panHu was restricted to cells from human, chimpanzee, gibbon and

Table 3. Species specificity of antibody CI-panHu

Species	Cell type	Reactivity* with: CI-panHu	Anti-HLA†
Human			
	Lymphocytes	98	97
Apes			
Chimpanzee	Hepatocytes	89	89
Gibbon	Lymphocytes	88	89
Old World Monkeys			
Rhesus	Kidney	86	88
African green	Kidney	10	60
Crab-eating macque	Kidney	0	0
New World Monkeys			
Marmoset	Lymphocytes	0	83
Others			
Wallaby	Buccal mucosa	0	0
Canine	Thymocytes	0	0
Sheep	Erythrocytes	0	0
Rabbit	Whole blood	0	0
Mouse	Splenocytes	0	0
Chicken	Whole blood	0	0

* Reactivity assessed by indirect immunofluorescence analysed by cytofluorograph. Numbers represent the percentage of cells staining.

† Monoclonal anti-HLA-A,B,C antibody W6/32.

Rhesus monkey (Table 3). Only very weak reactivity was detected with African green monkey cells and no reaction was found with cells from the crab-eating macaque, although both species belong to the Old World group, as does the Rhesus monkey. No reactivity was obtained with cells from any species phylogenetically more distant from man. The reactivity of the anti-HLA-A,B,C antibody W6/32 was assessed for comparison as it has been shown to react with cells from all Old World and some New World monkeys (Parham *et al.*, 1979).

Enzymic digestion of the CI-panHu antigenic determinant

Non-disruptive treatment of cells with the protease subtilisin (subtilopeptidase A) was found to markedly decrease the reactivity of the CI-panHu antibody (Table 4). Treatment of cells with the enzyme elastase also reduced CI-panHu binding. CI-panHu reactivity was not significantly affected by treatment of the cells with a number of other proteases, many of which reduced the binding of the anti-HLA-A,B,C antibody W6/32. Treatment of cells with the sialic acid-removing enzyme neuraminidase did not affect the binding of either antibody. Removal of sialic acid residues did not render the CI-panHu determinant more susceptible to removal by trypsin.

Table 4. Enzymatic digestion of CI-panHu antigenic determinant

Target cell line	Enzyme	Reactivity* with: CI-panHu	Anti-HLA†
THP-1 (monocytic)	No enzyme	91	81
	Trypsin	90	86
	Proteinase K	85	67
	Papain	85	67
	Pepsin	81	50
	Elastase	72	65
	Subtilisin	55	64
Colo 316 (ovarian)	No enzyme	73	ND‡
	Subtilisin	36	ND
Reh (lymphoid)	No enzyme	97	94
	Neuraminidase	98	99
	Neuraminidase/ trypsin	84	91

* Reactivity assessed by indirect immunofluorescence analysed by cytofluorograph. Numbers represent percentage of cells staining.

† Monoclonal anti-HLA-A,B,C antibody W6/32.

‡ ND, not done.

Molecular nature of the CI-panHu antigen

Identification of the CI-panHu antigen could not be achieved using techniques routinely employed in this laboratory for the analysis of monoclonal antibody-defined antigens (Fig. 3a). Thus, preforming of antigen–antibody complexes prior to cell

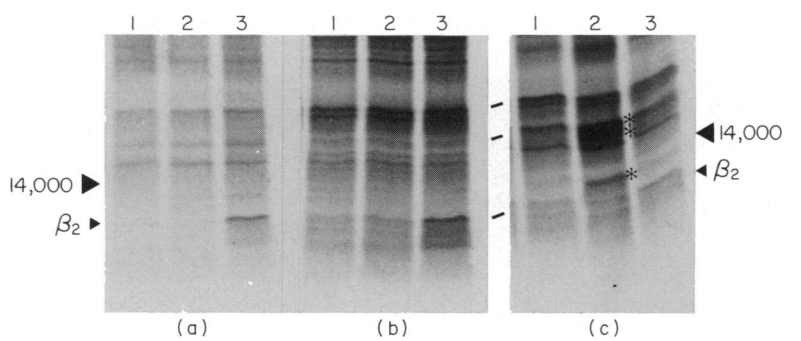

Figure 3. SDS–page analysis of protein immunoprecipitated by antibody CI-panHu. Fluorographs of one-dimensional SDS–PAGE analyses of proteins immunoprecipitated from ^{35}S-methionine-labelled Raji cell lysates (a), and from whole ^{35}S-methionine-labelled Raji cells using lysis buffer A (b) and lysis buffer B (c) as described in the Materials and Methods. Antibodies used for immunoprecipitation were normal mouse serum (Lanes 1), CI-panHu (Lanes 2) and W6/W32 (Lanes 3). Molecular weights were estimated by comparison with ^{14}C-labelled molecular weight markers (Amersham, Sydney, Australia; position of 14,000 MW protein shown) and the 12,000 MW β_2-microglobulin protein. Protein bands specifically precipitated by the CI-panHu antibody are indicated by asterisks.

lysis was attempted, followed by lysis under routine conditions (lysis buffer A). This too was unsuccessful (Fig. 3b). However, removal of deoxycholate from the lysis buffer used (lysis buffer B) enabled isolation of the antigen–antibody complexes formed. Subsequent SDS–PAGE analysis identified a 16,000 MW doublet as the major antigenic species (Fig. 3c). A 13,000 MW protein was also specifically immunoprecipitated. It is possible that the 13,000 MW protein is a biosynthetic intermediate in the synthesis of the 16,000 MW doublet. However, as these proteins were detected by reaction of antibody with intact cells, it seems more likely that these are independent protein species which are physically associated in some manner. Further, it is possible that either the 16,000 MW doublet or more probably the 13,000 MW protein is located intracellularly, and is present in CI-panHu immunoprecipitates by virtue of its association with the cell-surface exposed, CI-panHu-reactive species. The 12,000 MW β_2-microglobulin protein present in W6/32 precipitates by virtue of its association with HLA-A,B,C was detected under all experimental conditions. Identical results were obtained from similar analyses of the CI-panHu antigen from EBV-transformed B-lymphoblastoid cell lines (data not shown).

DISCUSSION

The CI-panHu monoclonal antibody detects an antigenic determinant expressed on the surface of all human cells, and as such is the first pan-human, cell-surface directed antibody described. The antigenic determinant detected is carried by a 16,000 MW cell-surface exposed protein doublet. No polymorphism in the expression of the CI-panHu antigen has been detected (number of individuals tested >300). As the population used in the experiments reported here was primarily Caucasian, it is possible that polymorphism of this antigen may exist in other racial groups.

The CI-panHu antigen is not associated with the HLA Class I antigens, as evidenced by the reactivity of the CI-panHu antibody with HLA-A,B,C-negative cells such as erythrocytes. The CI-panHu antigen is also not associated with β_2-microglobulin as the CI-panHu antibody reacts with the Daudi cell line which does not synthesize β_2-microglobulin.

Reactivity with the CI-panHu antibody was restricted to the higher primates; only certain species of Old World monkeys expressed the CI-panHu antigen. This range of expression is slightly more restricted than that of the HLA-A,B,C antigenic determinant detected by the monoclonal antibody W6/32, which is found in certain species of New World monkeys (Parham *et al.*, 1979) such as the marmoset (see Table 3). However, the phylogenetic range of the CI-panHu antigen is less restricted than that of the β_2-microglobulin antigenic determinant detected by the BBM.1 monoclonal antibody, which is only expressed in man and apes (Brodsky *et al.*, 1979). It is possible that expression of the CI-panHu antigen is polymorphic in species other than man, as is the case with the W6/32 antigenic determinant (Parham *et al.*, 1979).

The molecule(s) identified by the CI-panHu antibody comprised a 16,000 MW doublet as the major species, with an associated 13,000 MW protein component. The molecular composition of the CI-panHu antigen did not vary in different cells. On the basis of its molecular nature and tissue distribution, the CI-panHu antigen can be distinguished from other molecules reported in the literature. The immunoprecipitation data indicate that the CI-panHu antigenic determinant is sensitive to treatment with deoxycholate. It is, therefore, unlikely that the antigenic determinant will survive treatment with sodium dodecyl sulphate. This prevents the use of Western blotting procedures to identify the specific protein(s) carrying the CI-panHu antigenic determinant. This detergent sensitivity contrasts with the apparent insensitivity of the antigen to the many fixation procedures tested. In addition, the CI-panHu antigenic determinant is relatively resistant to a variety of proteases, more so than the HLA-A,B,C determinant recognized by the W6/32 antibody. This suggests that the CI-panHu antigenic determinant is more likely to be detectable in post-mortem tissues.

The 16,000 MW doublet identified by the CI-panHu antibody must presumably have a function that is cell-surface associated and that is necessary for cell function regardless of cell type. This molecule does not appear to be secreted as it has not been detected in intercellular or ductal spaces in tissues. The cellular distribution of this antigen, as visualized by immunoperoxidase staining, was principally cell-surface associated and no staining of basement membranes or related structures was detected. This makes it unlikely that this molecule forms part of any cytoskeletal network. It is possible that the CI-panHu molecule will prove to have some enzyme or receptor function essential to all cell types.

The CI-panHu monoclonal antibody has proved valuable as a positive control antibody in a variety of routine immunoassays (de Kretser *et al* 1985). In particular, its ubiquitous expression and its tolerance of many fixation procedures make this antibody an ideal choice as positive control for immunohistochemical staining experiments using monoclonal antibodies.

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